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Ferritin

BINDING OF BERYLLIUM AND OTHER DIVALENT METAL IONS*

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Rat liver homogenates in 0.1 M Tris, pH 7.5, were heated to 80 °C, cooled immediately, and centrifuged at 24,000 × *g*, and $^{7}Be^{2+}$ was added to the supernatant. Twenty-five per cent of the radioactivity was bound to a single protein. It was purified to homogeneity and identified to be ferritin as judged by different criteria. These were sucrose density gradient centrifugation, electrophoresis in polyacrylamide gel of the native or sodium dodecyl sulfate-treated protein, reactivity to antibodies, isoelectric focusing, and total amino acid composition. Comparative study of the ability of ferritin or apoferritin to bind Cd^{2+}, Zn^{2+}, Cu^{2+}, and Be^{2+} was conducted by using a gel equilibrium technique, Centifree micropartition technique, and microcentrifuge desalting technique. Ferritin could be saturated with Cd^{2+} or Zn^{2+} or Cu^{2+} but not with Be^{2+} even after 800 g atoms of Be^{2+} were bound. None of the bound Be^{2+} was dialyzable at 4 °C in 0.05 Tris acetate buffer, pH 8.5, but at pH 6.5 over 80% of the bound metal ion was dialyzed after 72 h. By contrast, apoferritin bound similar amounts of all four metal ions, some of which were dialyzable. By spectrophotometric titrations at pH 6.5 of Be^{2+} with sulfosalicylic acid (SSA), $Be_{K_D SSA}$ was calculated to be 5.0×10^{-6} M and by competition of sulfosalicyclic acid and ferritin for Be^{2+} the $Be_{K_D ferritin}$ was calculated to be 6.8×10^{-6} M.

Excessive intake of any cation is toxic. The toxic level and the expression of toxicity vary with the cation. For example in experimental animals, exposure to Pb causes, among other ill effects, inhibition of certain specific enzymes involved in the synthesis of heme (1). In addition Pb causes replacement of Fe in heme by Zn thus generating a nonfunctional Zn-protophorphyrin (1). In some instances, living systems respond to the toxic metal ions such as Cd^{2+}, Cu^{2+}, or Zn^{2+} by synthesizing metallothionein to sequester the detoxicant. The resulting protein binds a maximum of about 8 g atoms of the metal ions/mol (2).

One of the less commonly occurring metal ions in the environment is beryllium. This metal ion, atomic weight of 9.0122, is the lightest of the divalent metal ions. It is also one of the most toxic elements known. All forms of Be^{2+}, even at very low concentrations, adversely affect living systems (3). The molecular basis for the toxicity of Be^{2+} is as yet unknown. However, it is well established that some enzymes are inhibited by micromolar concentrations of Be^{2+}. Thus, out of great many enzymes tested, only three were inhibited at low concentrations of Be^{2+} (4, 5). These were alkaline phosphatase (6, 7), phosphoglucomutase (8, 9) and (Na^+K^+)-ATPase (10).

Our interest in Be^{2+} toxicity originated during our earlier investigations on the structure-function relationship of phosphoglucomutase from diverse origin (8). One of the parameters chosen for such a study was the effect of Be^{2+} on the activity of pure phosphoglucomutase from different species. The results showed that the rabbit muscle phosphoglucomutase binds a maximum of 1 g atom of Be^{2+} and such an enzyme metal complex is inactive. The binding of Be^{2+} to the enzyme is facilitated by chelating agents such as EDTA or cysteine because they do not chelate with Be^{2+} but remove other metal ions already bound to the enzymes (8). Subsequent studies showed that although partially purified phosphoglucomutase from rat liver was completely inactivated by micromolar concentrations of Be^{2+}, the enzyme activity in crude homogenates could be inhibited only partially (11). Indeed, at comparable concentrations of protein and Be^{2+}, the susceptibility to the inhibition of different preparations of liver phosphoglucomutase were: pure phosphoglucomutase > dialyzed extract > crude extract (11). This suggested that in a normal rat liver this enzyme is protected by at least two factors of different molecular weights; pure phosphoglucomutase contain neither, crude extracts contain both, and the dialyzed extracts contain only one, the large molecular weight protector(s).

Our search for the nondialyzable component led us to the isolation of a protein with a high molecular weight and capable of binding large quantities of Be^{2+}. In this paper it is identified as the iron storage protein, ferritin. Further, binding of Be^{2+} to ferritin is compared to that of Zn^{2+}, Cd^{2+}, and Cu^{2+}. Binding of Be^{2+} is in significantly greater amount than of other divalent metal ions. The majority of this Be^{2+} appears to bind to the iron core of ferritin. However, binding of Be^{2+} to the protein shell is also likely because apoferritin bound up to 160 g atoms of Be^{2+}. Speculation is made as to how the physical and chemical characteristics of beryllium would give rise to the observed binding.

EXPERIMENTAL PROCEDURES[1]

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[1] The "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-561, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Materials

$^{7}BeCl_2$ (carrier free, in 0.1 M HCl) and $^{109}CdCl_2$ (carrier free, in 0.1 M HCl) were purchased from Amersham. ^{64}Cu (10 mCi/mg copper, in 1N HNO_3) and $^{65}ZnCl_2$ (carrier free, in 0.5 M HCl) were purchased from New England Nuclear. Sephacryl S200, Sephacryl S300, Sephadex G100 (medium), Sephadex G-75 (medium), Sepharose CL-6B, and the molecular weight protein standards were purchased from Pharmacia. Horse spleen ferritin was purchased from Sigma and further purified as described below. Agar diffusion plates were purchased from Travenol Laboratories. PM30 filters, UM2 filters, and Centriffree micropartitions kits were purchased from Amicon. All other reagents were purchased from Sigma or Baker. Rabbit antiserum against horse spleen ferritin and sheep antiserum against human ferritin were kindly provided by Dr. I. Listowsky of Albert Einstein School of Medicine, New York, NY.

Methods

Eluates from columns were monitored at 280 nm. Whenever necessary protein concentration was measured by either the method of Lowry et al. (12) or Bradford (13) using bovine serum albumin as a standard and multiplying by appropriate correction factors. For the Lowry assay, protein values for ferritin and apoferritin were multiplied by 0.7 correction factor to get the best approximation. For the method of Bradford, protein values were multiplied by 0.73 for ferritin and by 0.82 for apoferritin to get the best approximation. The correction factors were determined by comparing the values obtained colorimetrically and by Kjeldahl nitrogen analysis (14).

Iron in ferritin or apoferritin was determined by atomic absorption spectroscopy on an IL 157 Instrumentation Laboratories spectrophotometer. Radioactivity was measured with a Beckman gamma counter. Protein samples for phosphate analysis were first digested in H_2SO_4 and were then analyzed for inorganic phosphate by the method of Bartlett (15).

Purification of Metallothionein. ^{109}Cd-thionein was purified from rat liver according to the procedure of Vander Mallie and Garvey (16) with some modifications. Six adult male albino rats (Charles River CD) were given daily injections of $CdSO_4$ in 0.85% NaCl (1.0 mg Cd/Kg body wt/per rat on the first day and 2.0 mg Cd/Kg body wt/per rat daily for the following 5 days). Another group of six rats was similarly injected with $BeSO_4$. Rats were sacrificed and their livers were homogenized in 5 volumes of 0.1 M Tris-HCl buffer, pH 7.4 containing 0.1 mg/ml phenylmethylsulfonylfluoride. The homogenate was centrifuged at 70,000xg for 30 minutes and the pellet was discarded. The supernatant was heated to 80° in a boiling water bath, cooled immediately and centrifuged at 27,000xg for 10 minutes. To the supernatant was added either $^{109}CdCl_2$ (for Cd injected animals) or $^{7}BeCl_2$ (for Be injected animals). To the radioactively labeled heat stable supernatants was added solid ammonium sulfate (24.3 g/100 ml) and after 30 minutes the precipitate formed was centrifuged at 27,000xg for 10 minutes and saved for radioactivity counting. To the supernatant was added solid ammonium sulfate (37.5 g/100 ml). After 30 minutes, the precipitate was centrifuged at 27,000xg for 10 minutes. The resulting pellet was dissolved in minimal amount of 5 mM Tris-HCl buffer, pH 7.4. To this solution was added cold acetone (-15°) to a final concentration of 50%. After centrifugation at 27,000xg for 10 minutes more cold acetone was added to the supernatant to yield a final concentration of 80% acetone. The solution was then centrifuged at 27,000xg for 10 minutes, and the pellet was dissolved in 0.5 mM Tris-HCl, pH 7.4. Further purificaiton was accomplished by Sephadex G-75 chromatography and DEAE cellulose chromatography as described by Vander Mallie and Garvey (16). Whenever required, apo-thionein was also prepared according to Vander Mallie and Garvey (17).

Purification of "Beryllium Binding Protein." Four adult albino rats (Charles River CD) were sacrificed, their livers removed, rinsed free of blood and homogenized in 5 volumes of 0.1 M Tris pH 7.4 containing 0.1 mg/ml of phenylmethylsulfonylfluoride. The homogenate was centrifuged for 30 minutes at 70,000xg at 4° and the pellet was discarded. The supernatant was heated to 80°C for 1 minute in a boiling water bath, cooled on ice, and then centrifuged at 27,000xg for 10 minutes. $^{7}BeCl_2$ (0.05 mc) was added to the heat stable supernatant and the solution was incubated overnight at 4°. To this solution was added solid ammonium sulfate (24.3g/100 ml) and after 30 minutes the precipitated protein was removed by centrifugation for 10 min. at 27,000xg. The precipitate was dissolved in 0.5 mM Tris pH 8.5 and applied to a Sepharyl S200 column (3x70 cm) previously equilibrated with the same buffer. The sample was then eluted with the same buffer. Fractions of 5 ml each were collected and assayed for protein and radioactivity. Fractions eluting with the void volume which were of constant specific radioactivity (CPM/A^{280nm}) were pooled and concentrated with an Amicon concentrator using a UM2 filter.

Purification of Rat Liver Ferritin. Ferritin was purified by the procedure of Linder and Munro (18) with slight modifications. Purification procedure was the same as that for the beryllium binding protein up to and including the steps leading to the heat stable supernatant. The heat stable supernatant was brought to pH 4.8 with dropwise addition of 0.15 M acetic acid. After 1 hour at 5° the denatured precipitate was centrifuged. Solid ammonium sulfate was added to the supernatant (31.3 g/100 ml). After 1 hour the precipitate formed was centrifuged at 24,000xg for 20 minutes. The pellet was dissolved in 0.05M phosphate pH 7.0 and applied to a Sephadex G100 (medium) column (3x70 cm) equilibrated and eluted with the same buffer. Ferritin, which eluted with the void volume, was concentrated as above.

Purification of Horse Spleen Ferritin. Ferritin purchased from Sigma was first dialyzed against 1000 volumes 0.01 M Tris pH 8.5 containing 0.5 mM EDTA overnight at 5° and applied to a Sepharose CL-6B column (3x70 cm) equilibrated and eluted with 0.01 M Tris pH 8.5 containing .5 mM EDTA. Five milliliter fractions were collected and the absorbance at 280 nm was monitored. Fractions in the major peak were pooled and dialyzed twice at 5° against 1000 volumes of 0.05 M Tris-HCl pH 8.5. Final preparations of electrophoretically pure horse spleen ferritin were stored in plastic vials at 5°.

Purification of Horse Spleen Apoferritin. Approximately 6 mg of horse spleen ferritin was dialyzed for 3 hours at 5°C against 2 liters of 1% thioglycolate in 0.1 M acetate buffer, pH 5.6. The thioglycolate was removed by further dialysis against 4 liters of 0.1 M acetate buffer, pH 5.6 for 3-4 hours at 5°, followed by dialysis against 2 liters of 0.05 M Tris-HCl, pH 8.5 with Chelex 100 to remove residual iron. In spite of this treatment, the final préparation contained between 24 and 30 gram atoms of iron per native apoferritin or 1-1.3 g atoms of iron per subunit. This is less than 1% of ferritin which contains 4000 g atom of iron.

Analytical Techniques. Visible and ultraviolet absorption spectra were recorded with a Cary 15 double beam spectrophotometer or a Beckman DU7 spectrophotometer. Ouchterlony immunodiffusion precipitation was done by applying 10 µl of protein samples (1mg/ml) to the surrounding wells and the antibody in the center well of the agarose gel plate. After precipitin bands became visible, the gel was washed extensively in 0.05 phosphate pH 7.4 in 0.85% NaCl; stained with 0.5% coomassie blue in methanol:acetic acid:water (5:1:5) and destained with the same solvent. Isoelectric focusing was conducted as described by Jeppsson (19). A 30 µg aliquot of protein was applied to precast LKB gels (5% acrylamide pH 3.5 - 9.5) electrofocused at 1500 V (20mM) for 1.5 hours and stained with coomassie brilliant blue R-250. Total amino acid composition of acid hydrolysates of the iron free proteins was determined with standard techniques using a Beckman amino acid analyzer (20). Sedimentation velocities were determined by sucrose density gradient centrifugation as described by Martin and Ames (21). A 2.0mg protein sample in 0.1 ml was layered on a 5.0 ml of a 20-50% sucrose gradient in 0.01 M phosphate, pH 7.0 and centrifuged at 70,000xg for 9 hours in an SW50.1 rotor. The gradient fractions were assayed for protein by absorbance at 280 nm.

Electrophoresis of native protein containing $^{7}Be^{+2}$ was done in duplicates on 5% polyacrylamide tube gels with bromphenol blue as the dye marker. One tube gel was stained with coomassie brilliant blue R-250 and the other cut into 4 mm sections. Each section was digested for 12 hours at 60° in capped vials containing 200 µl of 30% H_2O_2. To each of these vials was added 4ml of scintillation fluid (5.5g Permablend (Packard) per liter of toluene and one-third volume of Triton-X100) and counted for radioactivity.

Subunit molecular weight was determined by electrophoresis on SDS-polyacrylamide gels using the method of Laemmli (22). Proteins were dissolved in 0.05 M Tris-HCl pH 6.8 containing 2% SDS and 2% β-mercaptoethanol. Samples were heated for 5 minutes in a boiling water bath and electrophoresed with appropriate standards on 12.5% acrylamide slab gels. Staining of proteins was with coomassie brilliant blue R250.

Quantitation of Metal Binding Capacities of Proteins by Equilibrium Gel Filtration, Centrifree Separation, and Microcentrifuge Desalting Techniques. Equilibrium gel filtration of horse spleen ferritin and apoferritin was carried out using the method of Pieterson et al. (23). For each column run approximately 0.6 mg of protein was lyophilized and redissolved in 0.3 ml metal containing buffer. For Zn^{2+}, Cd^{2+}, and Cu^{2+} the metal-buffer consisted of 0.2 mM of the respective metal salt and 0.05 M Tris-HCl pH 7.4 buffer. For Zn^{2+} and Cd^{2+} radioactive tracer metal was used to monitor metal concentration. Concentrations of Cu^{2+} were determined by atomic absorption spectroscopy. In each case, protein was monitored by absorbance at 280 nm. Protein samples eluting with the void volume was pooled and assayed for protein by Lowry assay and for radioactivity by gamma counter. An aliquot of the pooled samples was dialyzed twice against 1000 volumes of 0.01 M Tris-HCl pH 7.4 to remove loosely bound metals. A second aliquot was dialyzed against 0.01 M Tris-HCl, pH 7.4 and 0.2mM EDTA to quantify tightly bound metal ions.

Centrifree micropartition technique (Amicon) was used to quantitate both the affinity and the maximal amount of metals that could be bound to ferritin. This method permits the separation of bound and free ligands. Ferritin or apoferritin from horse spleen were incubated for 15 minutes with various amounts of individual metal ions (Zn^{2+}, Cd^{2+} or Cu^{2+}). Remaining free metal was separated from the bound metal by centrifugation in the Centrifree filter for 15 minutes at 3100 rpm in a fixed angle clinical centrifuge. In all cases, metal concentration was quantified by atomic absorption spectrophotometry by radioactive tracer.

Solubility of $BeSO_4$ decreases with increase in pH. Thus the following procedure was employed to prepare Be-Buffers. To 0.05 mM unlabeled $BeSO_4$ (unbuffered) was added radioactive $^{7}BeCl_2$ (carrier free)and the specific radioactivity (CPM/mole Be) was determined. This solution was then adjusted to pH 6.3-6.5 with a dropwise addition of 0.1 M Tris base. The solution was then filtered through a microporous filter (0.45 µm. Amicon), the concentration of Be quantified by atomic absorption spectrophotometry and used immediately for the binding studies.

Horse spleen ferritins containing various amounts of bound Be^{2+} were prepared by using the microcentrifuge desalting technique of Hermorhorst and Stokes (24). A small amount of glass wool was placed in a 10 ml plastic syringe body and then packed with G-75 sephadex (medium) in 0.05 M Tris-HCl pH 6.5 and 0.005% azide to produce a bed volume of 5 ml. Columns were pre-centrifuged for 3 minutes at 1960 rpm in a swinging bucket type clinical centrifuge. Samples containing horse spleen ferritin were incubated with 0.05 M Tris-HCl pH 6.5 and various amounts of $^7BeSO_4$ (as prepared above). Incubation mixtures were concentrated approximately 10 fold by ultrafiltration (PM 30 filter) and the concentrated protein was applied to the pre-centrifuged column. The column was then centrifuged as before, but for 2 minutes. The eluted Be-ferritin was then quantified for the amount of $^7Be^{2+}$ bound. Protein was measured by absorbance at 280nm, utilizing extinction coefficients of 16.90 A^{280nm}/mg ferritin and 1.235 A^{280}/mg apoferritin. These values were obtained by parallel measurements by calorimetry using Lowry test and absorbance at 280 nm.

The beryllium chelator, sulfosalicylic acid (SSA), was used to remove Be^{2+} from Be-ferritins. ^7Be-ferritin, prepared as described above, was incubated with varying amounts of SSA and then the ^7Be-SSA was separated from $^7Be^{2+}$ which remained bound to ferritin by centrifugation in Centrifree filters for 15 minutes at 3100 rpm. Measurements of the radioactivity in the filtrate quantified the amount of Be^{2+} removed from ferritin by SSA.

The affinity of Be^{2+} for SSA was measured by spectrophotometric titrations. Varying amounts of $BeSO_4$ were added to SSA in 0.05 M Tris pH 6.5 buffer in a final volume of 3.0 ml and the increase in absorbance at 310 nm was measured with a Beckman DU-7 spectrophotometer.

RESULTS

Isolation of a High Molecular Weight "Be^{2+}-binding Protein"—As observed earlier (11), in crude liver extracts phosphoglucomutase is partially protected by dialyzable and nondialyzable factors. We first tested to see whether metallothionein could be one of the dialyzable factors, because it binds divalent metal ions such as Zn^{2+}, Cd^{2+}, and Cu^{2+} and its synthesis is induced by these metal ions. Accordingly, groups of rats were injected with Cd^{2+} or Be^{2+} and the isolation of liver metallothionein was attempted. Although our procedure for the induction and purification of Cd-thionein yielded the expected protein, injection of beryllium failed to induce metallothionein synthesis. When $^7Be^{2+}$ was added to the heat-stable supernatant of the liver homogenates of rats, 25% of the soluble $^7Be^{2+}$ appeared in the 0.4 ammonium sulfate precipitable fraction, a fraction in which metallothionein does not precipitate (17). The remainder of the $^7Be^{2+}$ was either free or bound elsewhere. A procedure developed for the purification of this Be^{2+}-binding protein is described under "Methods." The purified brick red protein eluted in the void volume of a Sephacryl S200 column, suggesting a molecular weight of at least 150,000. In polyacrylamide gel electrophoresis the native protein migrated as a single band containing the bound beryllium (Fig. 1).

Identification of the Be^{2+}-binding Protein as Ferritin—In 20–50% sucrose gradients the Be^{2+}-binding protein sedimented very closely to the horse spleen ferritin which was used as a standard. To test the identity of ferritin with the Be^{2+}-binding protein, rat liver ferritin was purified by the established procedure (18). In sucrose density centrifugation, rat liver ferritin and the Be^{2+}-binding protein sedimented as single proteins at 64.8 and 61.4 S, respectively. Visible and UV absorption spectroscopy indicated that both proteins had a broad absorbance below 400 nm, although the rat liver ferritin had a greater absorbance per milligram of protein. However, these differences disappeared when the proteins were treated with thioglycolate and dialyzed to remove iron. Both proteins had a subunit molecular weight of about 20,000 (Fig. 2) which is expected for ferritin. The two proteins had similar amino acid composition as well as electrophoretic patterns on isoelectric focusing gels (data not shown).

The identification of Be^{2+}-binding protein as ferritin was further established by their identical antigenic properties. Fig. 3 shows that the rat liver ferritin and Be^{2+}-binding protein formed continuous precipitin lines and formed a single spur when adjacent to horse spleen ferritin.

Thus, the two proteins were indistinguishable as judged by sucrose density sedimentation, sodium dodecyl sulfate-poly-

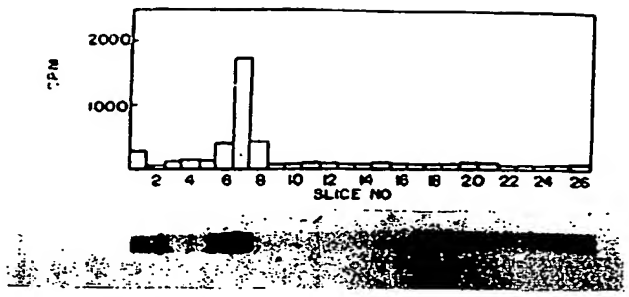

FIG. 1. **Native polyacrylamide gel electrophoresis of Be^{2+}-binding protein.** 80 μg of Be^{2+}-binding protein was electrophoresed in duplicate on 5% polyacrylamide tube gels. One gel was stained for protein and the other cut into 4-mm thick slices and the radioactivity counted.

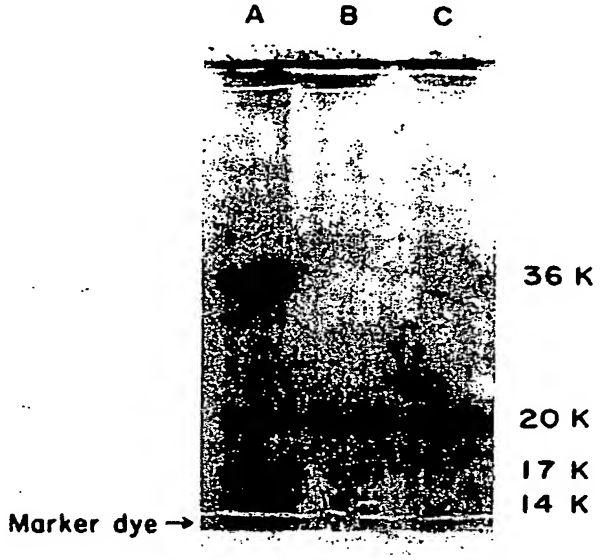

FIG. 2. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide).** Subunit molecular weights of Be^{2+}-binding protein (*right*) and rat liver ferritin (*center*) were determined to be approximately 20,000 each. *Left lane* indicates Be^{2+}-binding protein plus standards, glyceraldehyde-3-phosphate dehydrogenase (*36K*), myoglobin (*17K*), and lysozyme (*14K*).

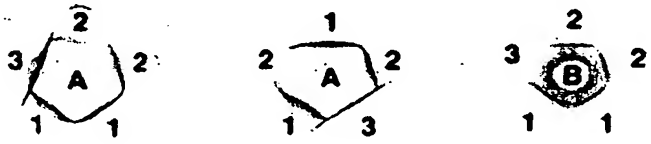

FIG. 3. **Immunological precipitation of ferritin and Be^{2+}-binding protein with antiferritin antibodies.** Ouchterlony gel diffusion plates reacting (*A*) rabbit anti-horse spleen ferritin or (*B*) sheep anti-human ferritin with (*1*) rat liver ferritin, (*2*) Be^{2+}-binding protein, and (*3*) horse spleen ferritin.

acrylamide gel electrophoresis, total amino acid composition, isoelectric focusing, and reaction with antibodies.

Quantitation of the Binding of Zn^{2+}, Cd^{2+}, Cu^{2+}, and Be^{2+} to ferritin and apoferritin—Once the Be^{2+}-binding protein was identified as ferritin, horse spleen ferritin was used for further studies. First, the equilibrium gel filtration method of Pieterson *et al.* (23) was employed to determine equilibrium binding of metals to ferritin or apoferritin at a 0.2 mM concentration of Zn^{2+}, Cd^{2+}, or Cu^{2+}. Fig. 4 shows a typical elution profile for ferritin run on the Zn^{2+} equilibrated column. Similar elution profiles were obtained for Cd^{2+} and Cu^{2+}. Fractions containing protein-bound metal ions were pooled and dialyzed against Tris-HCl buffer or Tric-HCl containing EDTA to distinguish the loosely and tightly bound metal ions (Table I). As seen, under equilibrium conditions ferritin bound 175 g atoms of Zn^{2+} and of Cd^{2+} but only 58 g atoms of Cu^{2+}. After dialysis against Tris-HCl, only 64 g atoms of Zn^{2+}, 83 g atoms of Cd^{2+}, and 34 g atoms of Cu^{2+} remained bound to ferritin. Dialysis in the presence of EDTA removed all but a small percentage of the bound metal, and different ferritin metal complexes were not distinguished on this basis.

In contrast to ferritin, apoferritin bound much less of Zn^{2+} and Cd^{2+}. The amount of Cu^{2+} bound to apoferritin (50 g atoms) was similar to that bound to ferritin (58 g atoms). Dialysis of metal-apoferritin complexes against Tris-HCl reduced all metals bound by about 50% and further dialysis against EDTA reduced Cd^{2+} and Cu^{2+} to 3 and 11 g atoms, respectively, similar to the levels remaining in ferritin. Zn^{2+}, however, was not significantly removed by EDTA.

In another series of experiments the equilibrium binding of Cd^{2+}, Cu^{2+}, and Zn^{2+} to ferritin or apoferritin was studied by Centrifree separation. This method is less time-consuming and allowed the variation in the concentration of metal ion incubated with the protein. Fig. 5 shows the Scatchard plots (25) for Cd^{2+} and Zn^{2+}. From these the dissociation constants, K_D (−1/slope), and the binding capacities, n (x − intercept/protein concentration) for Cd^{2+}, Cu^{2+}, and Zn^{2+} were calcu-

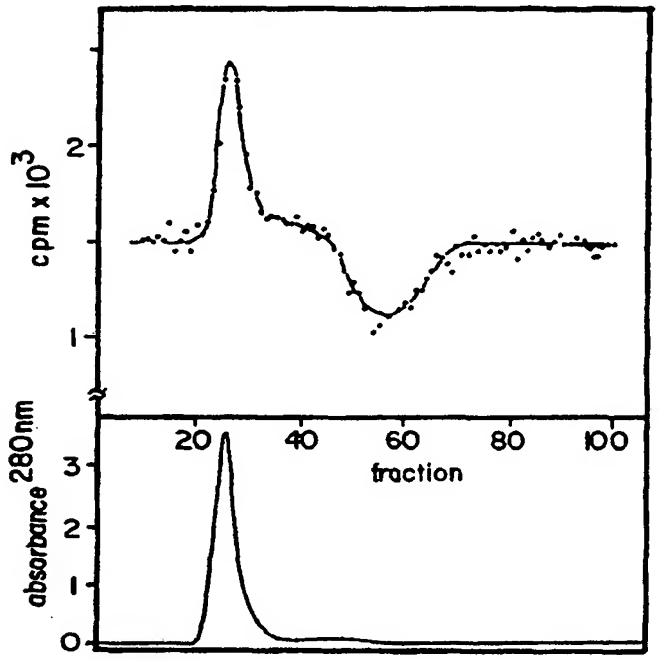

FIG. 4. **Zn-equilibrium gel chromatography of horse spleen ferritin.** The protein sample was dissolved in Zn-containing buffer, applied to a Sephadex G-75 (coarse) column, and eluted as described under "Experimental Procedures." Protein was monitored by absorbance at 280 nm and Zn concentration was measured by a ^{65}Zn tracer in a gamma counter.

TABLE I

Gram atoms of divalent metals bound to ferritin and apoferritin in equilibrium gel filtration

The experiment was done at the saturating concentration of metal ions, which for Zn, Cd, and Cu involved 0.2 mM divalent metal and 0.05 M Tris, pH 7.4.

	Ferritin						Apoferritin					
	Undialyzed		Buffer dialyzed		Buffer + EDTA dialyzed		Undialyzed		Buffer dialyzed		Buffer + EDTA dialyzed	
Zn	175	11	64	9	11	6	36	6			34	8
Cd	224	20	83	4	3	0.2	71	31	38	5	3	0.8
Cu	58	3	34	7	9	3	50	4	20	2	11	1

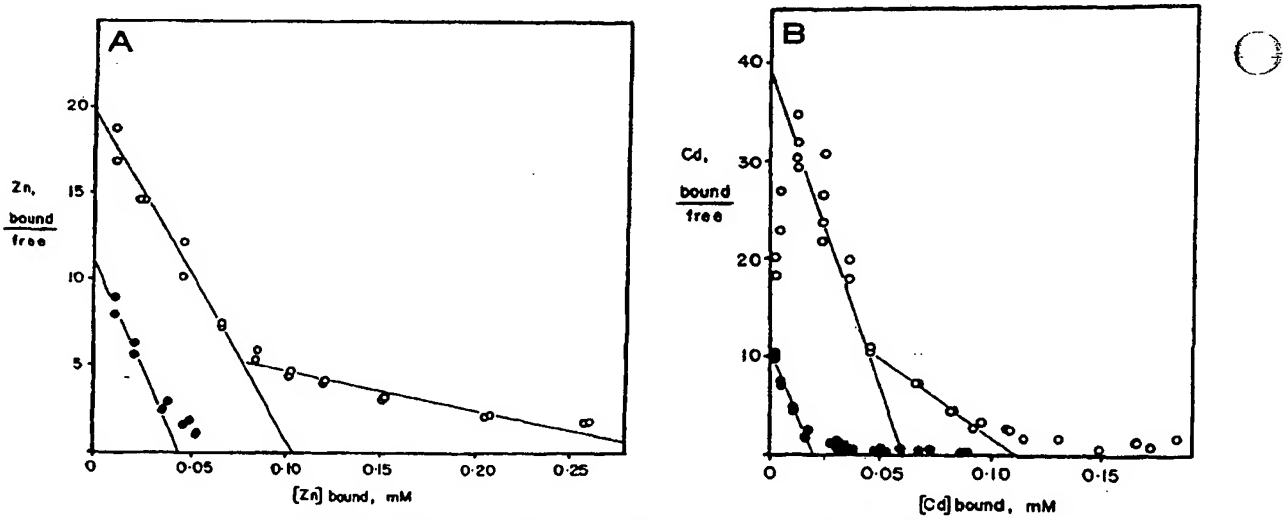

FIG. 5. **Scatchard analysis of divalent metal binding to ferritin (○) or apoferritin (●).** Metal ions tested for binding by the Centrifree method were Zn^{2+} (*A*) and Cd^{2+} (*B*). These plots were then analyzed for gram atoms of metal bound (n) and dissociation constant (K_D) as shown in Table II.

TABLE II

Binding parameter of divalent metals for ferritin and apoferritin as determined by micropartition followed by Scatchard plot

Metal	Ferritin		Apoferritin	
	n	K_D	*n*	K_D
		$\times 10^{-6}$ M		$\times 10^{-6}$ M
Zn	135.2	5.25	65.3	3.93
	251.0	44.1		
Cd	76.7	1.56	29.0	1.92
	61.6	6.11		
Cu	26.4	13.0	26.0	7.65

lated (Table II). As seen, ferritin has two classes of binding affinities for Zn^{2+} and Cd^{2+}, but only one for Cu^{2+}. For apoferritin, each of the three metals showed a single class of binding sites. The affinities of all the metals were of the magnitude of 10^{-6} M (Table II). Compared to apoferritin, ferritin bound more of Cd^{2+} and Zn^{2+}. As was seen in equilibrium gel filtration, ferritin and apoferritin bound similar amounts of Cu^{2+}. Some discrepancy between the results obtained by the Centrifree method (Table II) and by equilibrium gel filtration is apparent. In these cases, the Centrifree method is probably more reliable because the numbers represent extrapolation to the maximum number of binding sites.

The equilibrium gel filtration technique was unsuitable for Be^{2+}-binding studies because, as shown later, ferritin bound at least 800 g atoms of Be (see below) and at the protein concentration required for such studies, the amount of soluble Be^{2+} was insufficient to saturate the system. Therefore, binding of Be^{2+} to ferritin or apoferritin was studied by the Centrifree method. Fig. 6 shows the beryllium-binding data plotted with gram atoms on the *y* axis and total Be^{2+} concentration on the *x* axis. As seen, apoferritin bound a maximum of 160 g atoms of Be^{2+}, but ferritin was not saturated even after it bound 800 g atoms of Be^{2+}. When the binding of Be^{2+} was monitored by the microcentrifuge desalting method, it was expected to measure only those metal ions which were tightly bound (Fig. 6). Amounts bound as detected by this method were only slightly less than those observed by the Centrifree method. At 0.22 mM total Be^{2+}, ferritin bound 827 g atoms by the Centrifree method and 750 g atoms by the desalting method. In contrast, apoferritin under similar conditions bound 160 g atoms by Centrifree method and 60 g atoms by the desalting method. Measurement of iron showed no change in the amount of iron bound after Be was bound to ferritin or apoferritin. This ruled out a replacement of iron by Be^{2+} in these proteins.

To determine whether Be^{2+} either precipitated or dissociated after it was bound to ferritin, aliquots of three samples of ferritin containing 128, 377, and 599 g atoms of ^7Be were centrifuged in a sucrose gradient. For Be·ferritin containing 128 g atoms, all the $^7Be^{2+}$ migrated with ferritin during the centrifugation. For higher amounts of $^7Be^{2+}$ in ferritin (377 or 599 g atoms) about 20% of the ^7Be remained on top of the gradient and the remainder migrated with the ferritin. This suggests that some of the bound Be^{2+} dissociated due to dilution, and that the amount of dissociable Be^{2+} is dependent on the amount bound.

Quantitation of the affinity of Be^{2+} for ferritin and the dissociation of the bound Be^{2+} from ferritin was accomplished by using SSA,[2] a known reversible chelator for Be^{2+}.

Affinity of Be^{2+} for Sulfosalicyclic Acid—To carry out the above experiment, it was first necessary to determine the

[2] The abbreviation used is: SSA, sulfosalicylic acid.

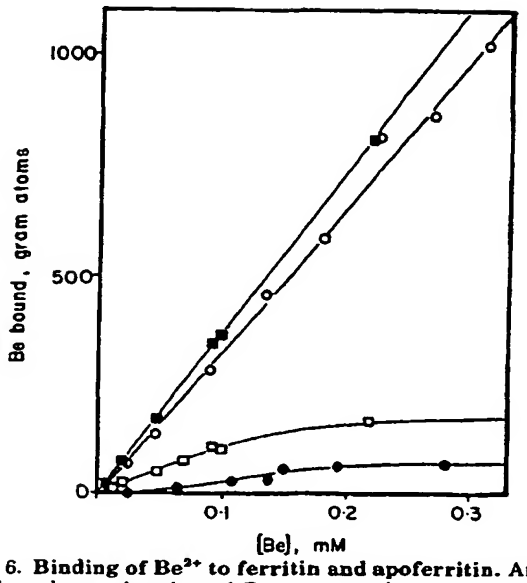

FIG. 6. **Binding of Be^{2+} to ferritin and apoferritin.** Amounts of Be bound as a function of Be concentration are measured by Centrifree technique (ferritin, ■; apoferritin, □) and by microcentrifuge desalting technique (ferritin, ○; apoferritin, ●).

dissociation constant of Be^{2+} for SSA at pH 6.5. Spectrophotometric titration of SSA with Be^{2+} (Fig. 7) revealed that binding of Be^{2+} decreases the absorbance at 300 nm and increases the absorbance at 310 nm.

The concentration of Be·SSA complex was determined by the equation:

$$[\text{Be·SSA}] = \frac{(\text{Abs}^{310} - \text{Abs}^{310}_{\text{SSA}})}{E^{310\,\text{nm}}} \quad (1)$$

where $E^{310\,\text{nm}}$ = molar extinction coefficient for Be·SSA = 4.088×10^3. The term $\text{Abs}^{310}_{\text{SSA}}$ is equal to the absorbance of free SSA at 310 nm expressed as a fraction of a total of 0.05 mM SSA. This is calculated as follows:

$$\text{Abs}^{310}_{\text{SSA}} = \text{Abs}_0{}^{310}\left(\frac{5 \times 10^{-5} - [\text{Be·SSA}]}{5 \times 10^{-5}}\right) \quad (2)$$

where $\text{Abs}_0{}^{310}$ = absorbance of 0.05 mM SSA alone at 310 nm = 0.842. Combining Equations 1 and 2 and solving for [Be·SSA],

$$[\text{Be·SSA}] = \frac{(\text{Abs}^{310} - \text{Abs}_0{}^{310})}{\left(E^{310\,\text{nm}} - \frac{\text{Abs}_0{}^{310}}{5 \times 10^{-6}}\right)} \quad (3)$$

Using Equation 3 and the absorbance values at 310 nm, the amount of Be·SSA complex and amount of free SSA are calculated.

From the Scatchard plot of the data (Fig. 8) the SSA_{K_DBe} at pH 6.5 was calculated to be 5.04×10^{-6} M.

Affinity of Be^{2+} for Ferritin—The competitive removal of $^7Be^{2+}$ by SSA was determined by adding increasing amounts of SSA to $^7Be^{2+}$·ferritin followed by the separation of the $^7Be^{2+}$·SSA from ferritin by Centrifree method. Fig. 9 shows the increase in $^7Be^{2+}$ removal from ferritin at increasing concentrations of SSA. Three ^7Be-ferritins were utilized. They contained 83, 335, and 698 g atoms of Be^{2+}, respectively. *Horizontal lines* represent the maximum amount of $^7Be^{2+}$ that could be removed from each ^7Be-ferritin. As can be seen in the three curves, SSA removed all but 20–40 g atoms of Be^{2+}. The affinity of Be^{2+} for ferritin was calculated by using the

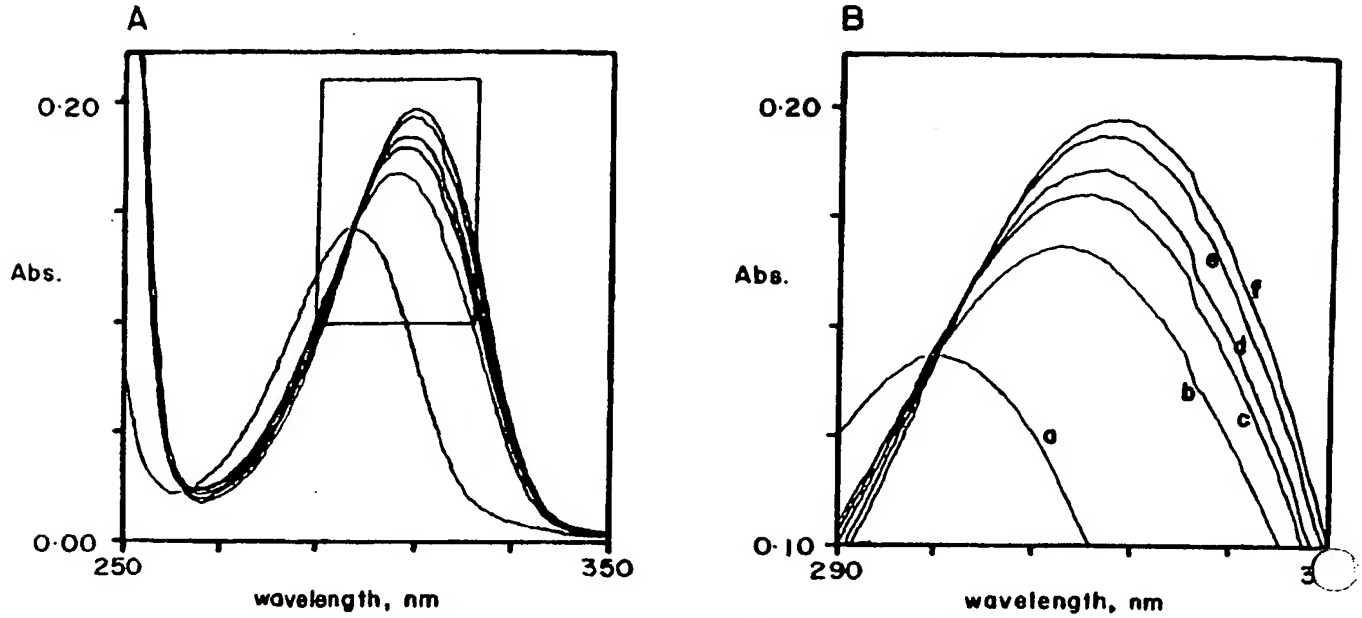

FIG. 7. **UV absorbance spectrum of SSA with increasing levels of Be^{2+}.** *B* is an enlargment of the rectangle shown in *A*. The buffer used was 50 mM Tris acetate, pH 6.5. The concentration of SSA was 0.05 mM in each sample. The final concentrations of Be^{2+} were 0 mM (*a*), 0.04 mM (*b*), 0.05 mM (*c*), 0.06 mM (*d*), 0.073 mM (*e*), and 0.10 mM (*f*). Absorbances at 310 nm were measured for quantitation of bound and free Be^{2+} (Fig. 8).

following relationships:

$$\text{Be·ferritin} \rightleftarrows \text{Be} + \text{ferritin}, \quad Be_{K_{D\text{ferritin}}} = \frac{[\text{Be}][\text{ferritin}]}{[\text{Be·ferritin}]} \tag{5}$$

$$\text{SSA} + \text{Be} \rightleftarrows \text{Be·SSA}, \quad Be_{K_A\text{SSA}} = \frac{[\text{Be·SSA}]}{[\text{Be}][\text{SSA}]} \tag{6}$$

SUM: Be·ferritin + SSA ⇄ ferritin + Be·SSA,

$$K_{eq} = \frac{[\text{ferritin}][\text{Be·SSA}]}{[\text{SSA}][\text{Be·ferritin}]} \tag{7}$$

Thus, the equilibrium expression (7) of the reaction carried out is the sum of two individual components (5 and 6). From these three reactions,

$$\frac{[\text{ferritin}][\text{Be·SSA}]}{[\text{SSA}][\text{Be·ferritin}]} = Be_{K_{D\text{ferritin}}} \cdot Be_{K_A\text{SSA}} \tag{8}$$

When half of the total Be^{2+} is removed from ferritin, the concentrations of ferritin and Be·ferritin are equal. Therefore, Equation 8 simplifies to:

$$\frac{[\text{Be·SSA}]}{[\text{SSA}]} = Be_{K_{D\text{ferritin}}} \cdot Be_{K_A\text{SSA}} \tag{9}$$

From the data for Be·ferritin containing 699 g atoms of Be^{2+} (Fig. 8) half-maximal removal of Be^{2+} was achieved at 3.7×10^{-5} M SSA. We now simplify the left half of Equation 9 and substitute the known values:

$$\frac{[\text{Be·SSA}]}{([\text{SSA}]_{total} - [\text{Be·SSA}])} = \frac{(1.55 \times 10^{-5}\ \text{M})}{(2.7 \times 10^{-5}\ \text{M} - 1.55 \times 10^{-5}\ \text{M})} = 1.35$$

The value of $Be_{K_A\text{SSA}}$ in Equation 9 is calculated as the inverse of the dissociation constant of Be for SSA as determined in the previous section:

$$Be_{K_A\text{SSA}} = \frac{1}{Be_{K_D\text{SSA}}} = 1.98 \times 10^5\ \text{M}^{-1}$$

We then substitute the values of (Be·SSA/SSA) and $Be_{K_A\text{SSA}}$ into Equation 9 and solve for the dissociation constant

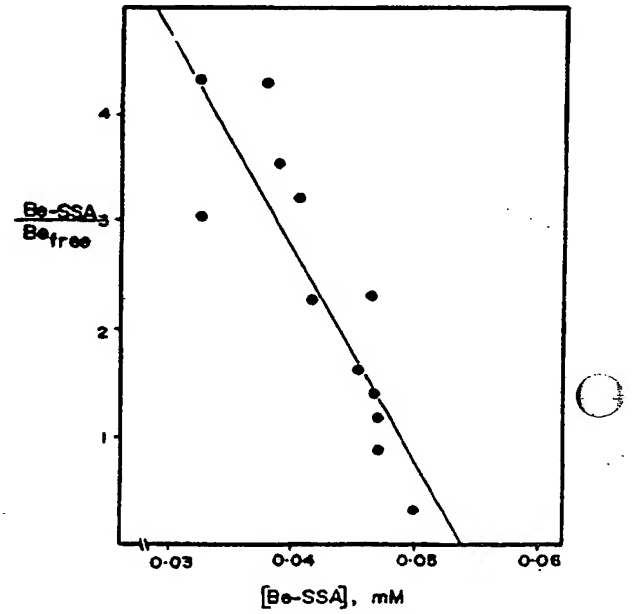

FIG. 8. **Scatchard plot of Be^{2+} binding to SSA.** Amounts of bound and free Be^{2+} were determined from spectrophotometric data (Fig. 7*A*) as described under "Results." From the least squares fit line the dissociation constant, $SSA_{K_D\text{Be}}$, was calculated to be -1/slope = 5.04×10^{-6} M.

of Be^{2+} for ferritin ($Be_{K_D\text{ferritin}}$):

$$Be_{K_D\text{ferritin}} = \frac{1.35}{1.98 \times 10^5} = 6.80 \times 10^{-6}\ \text{M}$$

Thus, by an indirect method the affinity of Be^{2+} for ferritin is calculated.

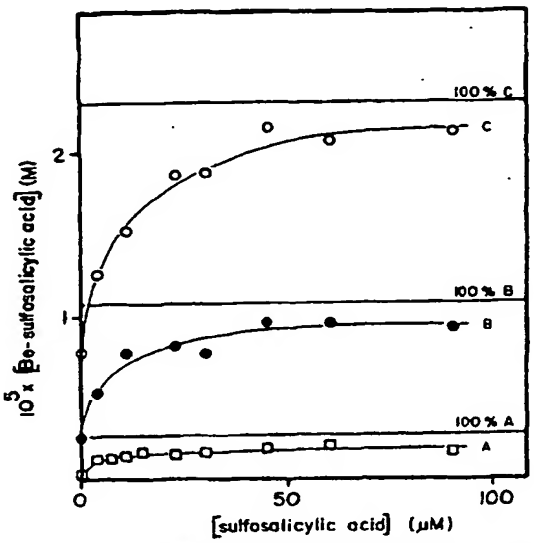

FIG. 9. **Removal of Be^{2+} from ferritin by SSA.** ^7Be-ferritins of three different Be^{2+} contents were made as described under "Methods": 83 g atoms of Be^{2+} (*A*), 335 g atoms of Be^{2+} (*B*), and 698 g atoms of Be^{2+} (*C*). ^7Be-ferritin (20 nM) and increasing levels of SSA (final concentration 0.1 mM) were incubated in a 0.5-ml final volume with 0.05 M Tris-HCl, pH 6.5, buffer for 2 h at 20 °C. Ferritin-bound Be^{2+} was then separated from SSA-bound Be^{2+} by the centrifree method. *Horizontal lines* represent the expected Be·SSA levels if all Be^{2+} were removed from ferritin.

DISCUSSION

The data presented here clearly establish ferritin as one of the major beryllium binding proteins in liver cytoplasm.

Consistent with the observations of Piotrowski and Szymanska (26), in our hands, injections of Be^{2+} did not induce the synthesis of metallothionein. In addition, apothionein did not bind Be^{2+} *in vitro*.[3] This was expected because divalent metal ions form mercaptides with metallothionein (2) and Be^{2+} does not form such bonds (27). Be^{2+}, however, does complex with phosphates and carboxylates (28, 29). Ferritin contains such groups. Indeed, carboxyl residues in ferritin bind Zn^{2+} and Tb^{2+} (30). Chasteen and Theil (31) have shown that vanadyl ions, VO^{2+}, compete with Zn^{2+}, Fe^{3+} Fe^{2+}, and Tb^{3+} for the binding to apoferritin. Similar EPR signals of VO^{2+} complexed with malonate or apoferritin further underscore the importance of carboxylate residues for metal binding (31). Beryllium also complexes with various nuclear nonhistone phosphoproteins (3) and other phosphate compounds (27). The well documented presence of phosphate in the iron core of ferritin (32) makes this protein well suited as a Be^{2+} chelator. We have observed in one of our preparations that horse spleen ferritin containing 1500 g atoms of iron had 322 g atoms of phosphate whereas its apoprotein containing 29 g atoms of iron had no detectable phosphate. Thus, most if not all of the phosphate in ferritin seem to reside in the iron core.

Beryllium salts in aqueous solution and at neutral pH form hydroxides. Unlike other group IIA elements, beryllium tends to form bonds of substantially covalent rather than ionic character. This has been attributed to the fact that beryllium has a relatively high nuclear charge coupled with a small atomic radius (33). Beryllium hydroxides are quite insoluble in water. Kosel and Neuman (27) have shown that a 50 mM aqueous solution of $BeSO_4$ titrated with NaOH formed insoluble $Be(OH)_2$ at pH 5.5 and above. This presents a problem when the binding of Be^{2+} to biological substrates is measured. Therefore, Parker and Stevens (34) used the SSA·Be^{2+} complex as a Be^{2+} donor to determine the affinity of Be^{2+} for nuclear acidic non-histone proteins. In calculating these affinities, however, the affinity of SSA for Be^{2+} appeared to have been ignored. It was therefore not surprising that SSA·Be and citrate·Be complexes gave very different results for the affinity of Be^{2+} to non-histone proteins. The data presented here show SSA_{K_DBe} to be 5.04×10^{-6} M. In these calculations, a 1:1 binding of SSA to Be^{2+} is assumed. Das and Aditya (35) have shown 1:1 binding of SSA to Be^{2+} at pH 4.5 and 4.0. They have reported that at higher pH (between 9 and 11), SSA binds to Be^{2+} in a 2:1 ratio. Since our pH (6.5) was much closer to 4.5, we concluded that the binding our experiments was primarily in a 1:1 ratio. The fact that our calculated SSA_{K_DBe} (5.04×10^{-6} M) was much smaller than that determined by Das and Aditya (5.4×10^{-5} M at 0.05 ionic strength and 29.5 °C) may be due to increased ionization of SSA at the higher pH. Using our value for SSA_{K_DBe}, the affinity of Be^{2+} for ferritin was determined.

The large amount of Be^{2+} bound to ferritin is especially noteworthy. Ferritin bound greater than 800 g atoms of Be^{2+} under both equilibrium and dialyzed conditions. This large amount of tightly bound Be^{2+} bound is probably associated with phosphate (322 g atoms) which is present in the iron core. We were also able to get as high as 1200 g atoms of Be^{2+} bound to ferritin at a higher total Be^{2+} concentration. The high levels of Be^{2+} bound to ferritin under these conditions may be an artifact if a loss of subunits from the ferritin shell has occurred. If the calculation of molar quantity of ferritin molecules is erroneously low due to this damage, then it would appear that the gram atoms of Be^{2+} were greater than were actually bound to each ferritin molecule. Perhaps, Be^{2+} tightly associated with the core disrupts the iron oxyhydroxide-phosphate lattice and results in the partial breakdown of the ferritin molecule.

Apoferritin binds substantially less Be^{2+} than did ferritin and of the 160 g atoms bound only 50–60 are tightly bound. Since the percentage of tight binding Be^{2+} is so much lower for apoferritin than for ferritin, one would suspect a different mode of binding. Thus rather than binding to the residual iron (29 g atoms) or phosphate (none detected) of apoferritin, we suspect that Be^{2+} is bound to the carboxyl residues of aspartic or glutamic acid or the hydroxyls of tyrosine on the protein shell.

Binding of Zn^{2+}, Cd^{2+}, and Cu^{2+} to ferritin or apoferritin is compared to that of Be^{2+}. In some instances Scatchard plots are employed despite the limitations of this method (36) because it permitted better comparison of our data with those of Macara *et al.* (37) who studied the binding of various divalent metal ions such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, and Tb^{2+} to apoferritin. It was found for all these metal ions except Mn^{2+}, that 2–3 metal ions/subunit were tightly bound and 3–4 metal ions/subunit were loosely bound. For Mn^{2+} there is only 0.5 metal ion tightly bound and 2 metal ions loosely bound per subunit. Our results for the binding of Zn^{2+}, Cd^{2+}, and Cu^{2+} to apoferritin were very similar to the results of Macara *et al.* (37). Although we obtained a hyperbolic Scatchard plot for these metals as did Macara *et al.*, we have only determined values for what are the most tightly bound metals. Our results for the binding of Zn^{2+}, Cd^{2+}, and Cu^{2+} to ferritin show some different trends. Zn^{2+} and Cd^{2+} binding to ferritin showed an increased number of low affinity sites, but there was also about a 50% increase in the high affinity sites compared to apoferritin. Cu^{2+} binding is not increased for ferritin as was the case for the other metals. Harrison *et al.* (42) has reported that ferritin and apoferritin have the same

[3] D. J. Price and J. G. Joshi, unpublished observations.

number of high affinity sites for Zn^{2+}. Our results suggest that during the formation of ferritin from apoferritin some new high affinity Zn^{2+} sites are created.

The binding of Be^{2+} to apoferritin was of particular interest when compared to the binding of other metals. We show that apoferritin binds a total of 6.7 g atoms/subunit and of these only 2.1–2.5 are tightly bound. Thus, our findings of loosely and tightly bound Be^{2+} to apoferritin are in close agreement with those observed for other metals by Macara *et al.* (37). It is therefore likely that in the ferritin molecule the carboxylate residues are required for Be^{2+} binding just as they have been shown for the chelation of Zn^{2+} (38).

The binding of Be^{2+} to ferritin appears to be different from that of other metals. Although all the metal ions except Cu^{2+} showed binding which could be attributed to the iron core, beryllium binding was by far the highest in amount and affinity. This binding could be to either phosphate or ferric hydroxide components of the core. However, since phosphate is known to bind Be^{2+}, the binding of Be^{2+} to the phosphates of the core is the most likely possibility. Experiments are now in progress to test this possibility. To quantify the tightness of the binding of Be^{2+} to ferritin, we have measured the removal of the metal ion from ferritin by a known chelator, SSA. This technique is analogous to that of Vallee and Coombs (39) who estimated the binding of Zn^{2+} to alcohol dehydrogenase.

The sequestering of Be^{2+} by ferritin is significant not only because of the large amount bound and the tightness of binding, but also because the bound metal would have only limited accessibility to other proteins. The x-ray data of Banyard *et al.* (40) has shown that the only access to the ferritin core is through pores of 10 Å diameter of the 4-fold axis of the protein shell. Thus, formation of beryllium complexes in the iron core of ferritin would make Be nonaccessible to other proteins which might have a higher affinity for beryllium. In particular, enzymes which are susceptible to inhibition by beryllium could be protected by this sequestration.

The tissues shown to accumulate highest amounts of injected beryllium, liver and spleen (41), are also rich sources of ferritin (42). We have reported previously that Be^{2+} injected into rats is sequestered at least in part by ferritin (43) and that *in vitro* ferritin can reactivate alkaline phosphatase, phosphoglucomutase, and (Na^+K^+)-ATPase which are inhibited by micromolar concentrations of Be^{2+} (44). This is so despite the fact that, as shown in this paper, $Be_{K_{d\,ferritin}}$ is 6.8×10^{-6} M. A partial explanation for this apparent discrepancy is that these studies were done at pH 7.4 and at alkaline pH the Be^{2+} bound to ferritin is nondialyzable. Presumably, like the ferric hydroxy phosphate sequestered by ferritin, Be^{2+} is also internalized although this remains to be established. In addition, under reactivating condition used for enzyme studies the concentration of ferritin is greater than that of the enzymes, and compared to the enzymes ferritin binds far more Be^{2+}. It is not claimed that ferritin is the only Be^{2+} binding protein capable of a protective effect. Nevertheless, based on the properties of beryllium binding shown here, ferritin may function as a natural detoxicant for beryllium.

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